WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 7.523
Volume 6, Issue 17, 846-857. Research Article ISSN 2277–7105

METHOD VALIDATION AND SIMULTANEOUS DETERMINATION OF TWO BIO-ACTIVE MARKER COMPOUNDS IN PONGAMIA PINNATA: SEED AND KARANJ OIL BY UPLC-MS

N.M.A. Rasheed[1], K. Nagaiah[1]*, G. Sowmya Srividya[1] and M.V.N. Kumar Talluri[2]

[1]Organic & Biomolecular Chemistry, CSIR-Indian Institute of Chemical Technology, Tarnaka, Hyderabad-500007.

[2]National Institute of Pharmaceutical Education and Research (NIPER), Balanagar, Hyderabad, 500037.

Article Received on
22 Oct. 2017,

Revised on 13 Nov. 2017,
Accepted on 04 Dec. 2017,

DOI: 10.20959/wjpr201717-10395

***Corresponding Author**
Dr. K. Nagaiah
Senior Principal Scientist, Organic & Biomolecular Chemistry, CSIR-Indian Institute of Chemical Technology, Tarnaka, Hyderabad-500007.

ABSTRACT

Seed of *Pongamia pinnata* (Linn.) Merr known as "Karanj" widely used in traditional system of medicine. Oil from karanj seeds contain flavonoids which possess diverse pharmacological properties. The seed oil was subjected to karanj oil-liquid extraction with methanol to isolate karanjin and pongamol which are active principle of karanj. The oil mainly used for skin diseases such as psoriasis, eczema and vitiligo etc. A validated reversed-phase ultra-performance liquid chromatography method with photodiode array detector was successfully developed for the first time report to simultaneously determine two active compounds karanjin and pongamol in karanj seed extract and its oil. Separation carried through a BEH C18 column by gradient elution using 0.1% formic acid in water (v/v): methanol: acetonitrile at 0.2 mL/min. Significant linear correlations were found between component concentrations and specific chromatographic peak areas. The % R.S.D. values found by the method indicating precision, range and recovery to be good. Thus the proposed validated method can be applied as a reference standard for quality control analysis commercial samples of karanj oil.

KEYWORDS: Karanj oil, *Pongamia Pinnata*, UPLC, Karanjin, Pongamol.

INTRODUCTION

The seeds of *Pongamia pinnata* (Linn.) Merr, syn. *Pongamia glabra* vent. (Family-Leguminosae) mostly used in traditional Indian system of medicine particularly in Unani and Ayurveda and popularly known as karanj in urdu, karanja in hindi. It is a medium sized glabrous tree spread throughout India at an altitude of 1200 m. Macroscopically seed of karanj appear to be one and rarely two elliptic or somewhat reniform in shape, testa as reddish leathery. Transverse section of karanj seed shows testa as composed of a layer of palisade like outer epidermis, filled with brown pigment, covered externally with a thick cuticle, thick-walled parenchyma cells of two to four layers, and a number of parenchyma cells containing brown pigment.[1] All parts of *P. pinnata* are therapeutically useful for treating tumors, piles, skin diseases, wounds and ulcers.[2] The names and structure of the chemical principles isolated from *P. pinnata* is given in Fig-1.

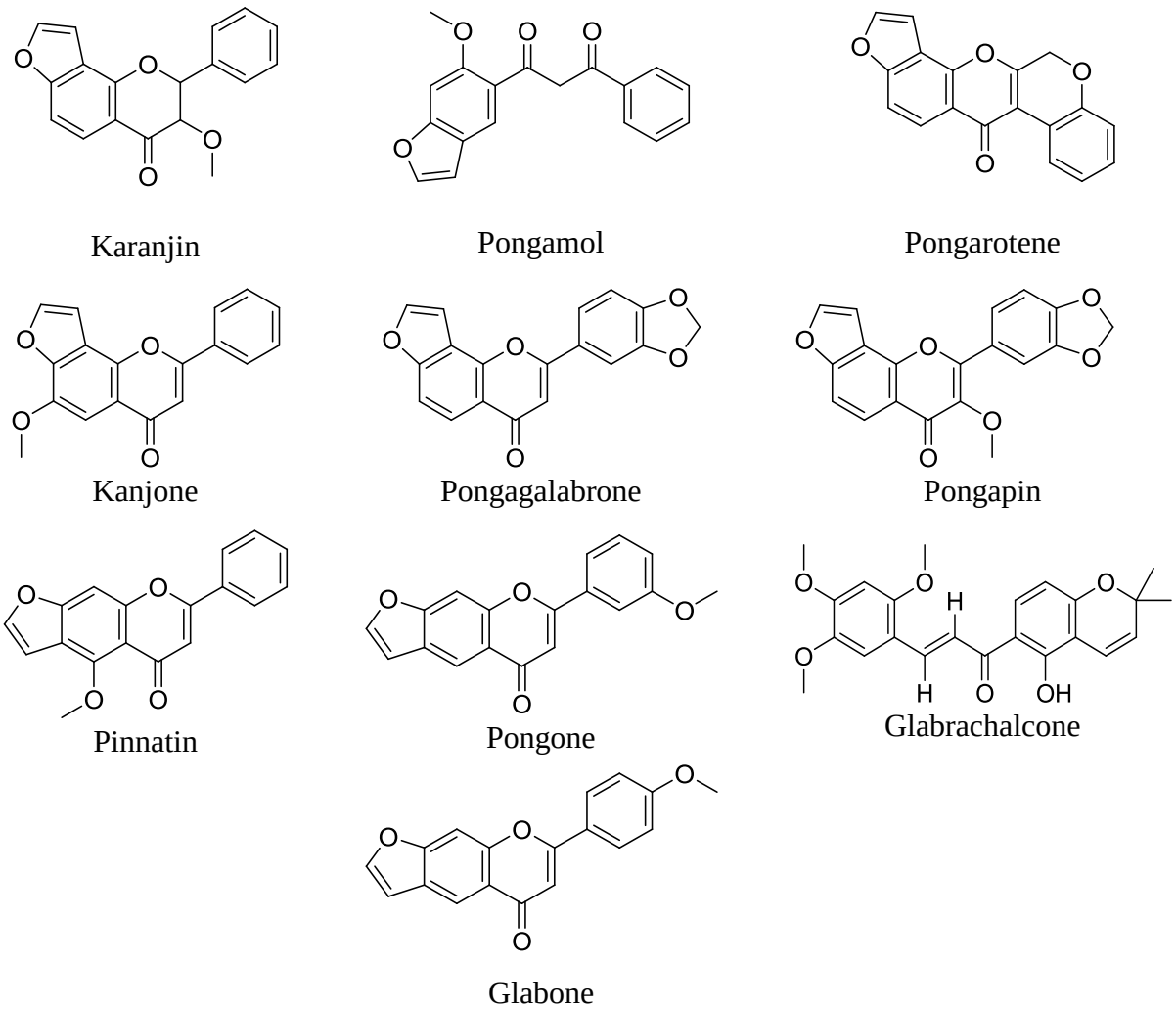

Fig. 1: Chemical structures of constituents of *P. Pinnata* L.[3-5]

Karanj oil from seeds contain two important compounds karanjin and pongamol, both are bioactive compounds with important biological attributes. Seed reported to possess anthelmintic activity, effective in treatment of leprosy, chronic fever, also helps in piles, relives liver pain, heals ulcers.[6] Seed extracts possesses antiinflammatory activity,[7] anti-oxidative, anti-ulcerogenic properties, analgesic and hypoglycaemic,[8] also useful in rheumatism arthritis and scabies[9] and it is used to treat dermatitis of pet animals[10-11] and gastroprotective.[12] Various herbal remedies separately or along with combination were recommended in different medical conditions for the cure of skin diseases such as psoriasis, eczema and vitiligo.[13] The oil reported to be antihelmintic, styptic and recommended for opthalmia, leprosy, ulcers, herpes and lumbago. Its oil utilized as a source of biodiesel.[14-15] Karanj oil reported to contains 5–6% flavonoids, the main active constituents found are karanjin, (a furano-flavonoid) and pongamol (a diketone) which possess various pharmacological properties[16] and their isolation in pure form and efficient quantitative method with more precise and accurate content in seed extracts, seed oil and also commercially available oil samples at present a great need.[17] Karanjin showed pesticidal and insecticidal properties.[18] Dermal absorption of karanjin from *Jatyadi Taila* an oil based Ayurvedic drug.[19] The presence of karanjin, pongamol, pongagalabrone, pongapine, pinnatine and kanjone in *Pongamia pinnata* which are mainly responsible for various pharmacological activity.[20] (Fig-1)

Another use of karanjin oil as starting material for biodiesel, the manufactures do not seem to remove karanjin, pongamol etc for biodiesel which can be more useful for medicinal purpose as described above. Karanj oil also used to light lamps, where karanjin, pongamol and other compounds and other active principle**s** are not required. Biodiesel is prepared by trans-esterification of karanj oil by heating with NaOH or KOH and methanol. Karanjin is not desirable in biodiesel because it corrodes the engine parts. If karanjin is removed before, the biodiesel is efficient. On the other hand, the crude karanjin and pongamol enriched oil as shown in the present study**,** could be used in skin ointments.

Earlier few methods were reported by Gore and Satyamoorthy, Shejawal *et al*.[11,21] which are based on conventional HPLC for karanjin only and for both compounds based on dual wavelength detection and are not validated. The objective of this study was to develop a validated UPLC method for simultaneous determination of karanjin and pongamol as per ICH guidelines[22] at a single wavelength. The seed oil was subjected to karanj oil–liquid

extraction with methanol to isolate pure compounds of karanjin and pongamol[13] and preparation of enriched oil for topical application in the treatment of skin diseases. Till now no method was reported through UPLC for the estimation of karanjin and pongamol in commercial or marketed samples to the best of our knowledge. Thus efforts were made to develop a validated RP-UPLC method by PDA.

MATERIAL AND METHODS

All HPLC grade reagents were used. HPLC-grade methanol, acetonitrile, water, formic acid, orthophosphoric acid, triflouroacetic acid were used. Marketed Samples of karanj oil such as Vyas karanj oil and Cold pressed pure karanj oil make Soul-centric was procured and karanj seeds were procured from the pharmacy, Central Research Institute of Unani Medicine, Hyderabad and one from local region. Karanjin and pongamol (figure 1) are the pure isolated compounds from *Pongamia pinnata* and characterized through spectrophotometric methods and used as reference standards. These pure compounds available in our laboratory. Karanj oil is enriched with respect to karanjin etc., by oil/liquid extraction with methanol or incorporating technical karanjin into oil. Syringe filter PTFE membrane of 0.22 µm pore size, dia. 25mm (Axiva).

Standard preparation

Standard stock solutions of karanjin and pongamol were made with 10mg in 10ml with methanol to obtain 1000 µg/ml each separately. Aliquots of stock solutions were prepared by dilution to obtain required concentrations of 10, 20, 30, 40, 50 µg/ml and 2µl was injected.

Sample preparation

Samples of karanj oil and seeds were accurately weighed one gram each and transferred in 100 ml beaker, and extracted with 10ml methanol by ultrasonic extraction method for 15 min. The sample extracts filtered through syringe filter PTFE membrane of 0.22 µm pore size, stored and used for analysis.

Chromatographic conditions

The mobile phase before delivering was filtered through 0.22 µm, PTFE filter and sonicated with the help of ultrasonicator for 15 minutes. The analysis was carried out under gradient conditions at a flow rate of 0.2µL/min at temperature 40^{o}C and recorded at 350nm wavelength.

Instrument conditions

Acquity UPLC-H class, a quaternary pump, and PDA detector was used. Analyses were carried on Acquity BEH C18 reversed-phase analytical column (2.1 × 100 mm, 1.7 µm), flow rate of 0.2 mL/min, at 350 nm wavelength, injection volume 2 µL, column temperature 40°C. The mobile phase A (0.1% formic acid in water), solvent B (methanol) and solvent C (acetonitrile) filtered through a 0.22 µm membrane filter; gradient elution as follows: 0 min 40% A, 40% B, 20%C; 1 min 37% A, 48% B, 15%C; 8 min 34% A, 56% B, 10%C; 9 min 30% A, 60% B, 10%C; 11 min 25% A, 65% B, 10%C; 15 min 40% A, 40% B, 20%C; 18 min 40% A, 40% B, 20%C. The data were collected and analyzed using Waters Software Empower 3.

RESULTS AND DISCUSSIONS

Method development and optimization

Using isocratic elution, all the compounds cannot be effectively separated; thus, gradient elution was used throughout the study. Suitable wavelength selected for simultaneous determination of karanjin and pongamol in the range of 200-400 nm and both had good absorbance at 350 nm. The chromatographic variables were also optimized, including column temperatures and column types. Optimum separation was eventually achieved with the reversed-phase Waters Acquity UPLC™ BEH C18 2.1 x 100 mm, 1.7 µm, at 40°C temperature. To obtain chromatograms with well-resolved peaks and comfortable analysis time/run, chromatographic conditions such as column temperature, type of reversed-phase column, extraction solvent, detection wavelength, and mobile phase along with buffer were optimized. In this study, methanol, acetonitrile and water along with a small amount of formic acid as buffer was added to the mobile phase. The acid inhibited the ionization of acidic compounds in the samples to improve peak shape and restrain peak tailing. Subsequently 0.1% formic acid in the mobile phase significantly improved the retention behavior and peak shape of the different components in karanj oil samples. The following optimized chromatographic conditions were obtained for analysis as mobile phase A: (0.1% FA in water) and B: (methanol) and C: (acetonitrile). Gradient conditions for elution as 0 min 40% A, 40% B, 20%C; 1 min 37% A, 48% B, 15%C; 8 min 34% A, 56% B, 10%C; 9 min 30% A, 60% B, 10%C; 11 min 25% A, 65% B, 10%C; 15 min 40% A, 40% B, 20%C; 18 min 40% A, 40% B, 20%C. The flow rate was 0.2mL/min, and the column temperature was 40°C. A typical UPLC chromatogram as shown in figure 2. The retention times for the compounds detected in the Karanj oil samples were at karanjin (t_R 7.3 min) and pongamol (t_R

13.6 min). Upon LCMS analysis, karanjin and pongamol was detected in the positive mode were identified and confirmed in standard solution and in the Karanj oil samples through LCMS data.

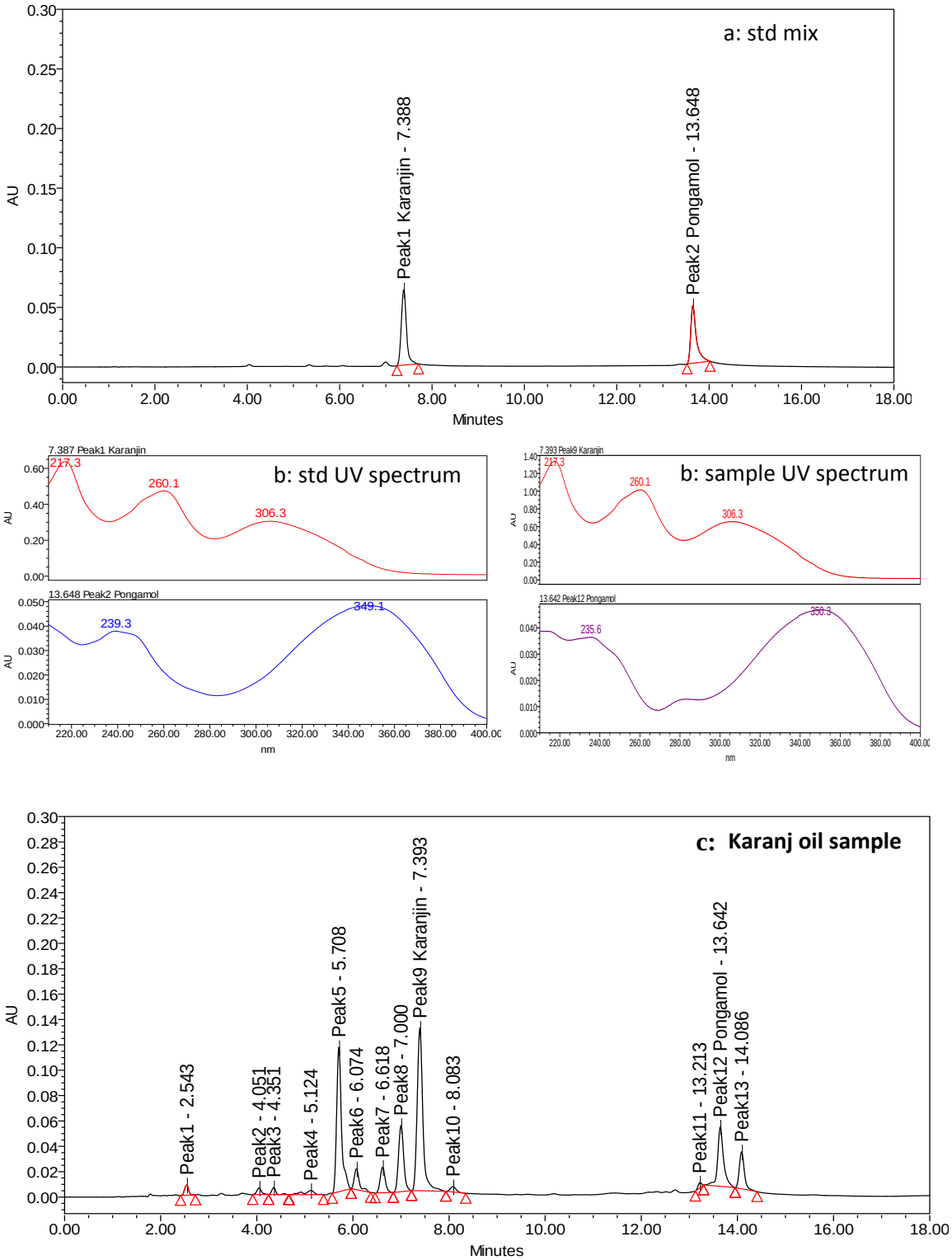

Fig. 2. UPLC chromatogram of (a) standard mixture, b) UV Spectrum of karanjin and pongamol of standard and sample c) karanj oil sample.

System suitability

To ensure adequate performance of the chromatographic system, we evaluated the retention time (tR), Resolution (RS), number of theoretical plates (N), and tailing factor (T) using standard solution for karanjin and pongamol such as retention time (tR), Resolution (RS), number of theoretical plates (N), and tailing factor (T), all parameters were good and in acceptable limits.

Linearity, limit of detection and limit of quantitation

After establishing the optimum conditions, method validation was performed. Good linear correlation and high-sensitivity under these chromatographic conditions were confirmed by the correlation coefficients (R^2), limits of detection (LOD), and limits of quantitation (LOQ)

The linearity calibration curves were plotted based on at least five calibration points performed in triplicate. The correlation coefficient R^2 and linear regression equations were analyzed. Results of regression analyses and the calculated correlation coefficients (R^2) indicated good linearity within concentration ranges for both the investigated compounds at the wavelengths at 350 nm as given in table 1.

Table 1: Statistical results of linear regression equation analysis in the determination of the two investigated compounds.

S.no	Parameter	Karanjin	Pongamol
1	Linear Range (µg/ml)	10-50	10-50
2	Regression equation	y = 96615x - 699.7	y = 93440x - 64619
3	R^2	0.998	0.995

Limits of detection and limits of quantitation under the present chromatographic conditions were defined as the lowest concentration with the signal-to-noise ratio of 3 and 10 as criteria, respectively. The LODs for karanjin and pongamol was 0.25 and 1.41 µg/ml respectively and LOQs for karanjin and pongamol was 0.86 and 4.70 µg/ml respectively.

Precision, repeatability and recovery data

Precision was evaluated by analyzing standard samples. Thus, five individual sample solutions were analyzed. For each compound the RSD of the mean was calculated and ranged from 0.13% to 0.60% for intraday precisions. Hence the proposed method was found to be precise. The results are shown in table 2. The recoveries of karanjin and pongamol were assessed by spiking known amount each at three different levels ranging 50µg, 100 µg and 150 µg with respect to concentration of karanjin and pongamol in karanj oil samples found

and further analyzed by UPLC. All estimations were repeated thrice (n=3) and the relative standard deviations were calculated. The recovery range and %RSD for karanjin and pongamol were found to be 96.94-99.32% and 100.55-105.25% with %RSD < 0.0018% respectively as showed in table 2.

Table 2: Precision, Repeatability and Recovery of two compounds.

	Karanjin	Pongamol
Repeatability (Mean ± %RSD) (n=3)	11873382± 0.16	14939121± 0.13
Recovery (Mean ±%RSD) (n=3)	99.32 ±0.0018	100.55 ±0.0013

Precision	**Karanjin**		**Precision**	**Pongamol**	
Conc. µg/ml	**Intraday (n=3)**	**Inter day (n=3)**	**Conc. µg/ml**	**Intraday (n=3)**	**Inter day (n=3)**
	Mean ± %RSD	**Mean ± %RSD**		**Mean ± %RSD**	**Mean ± %RSD**
10	97913 ± 0.53	97166 ± 0.86	10	97913 ± 0.53	97166 ± 0.86
20	191663 ± 0.13	192235 ± 0.99	20	191663 ± 0.13	192235 ± 0.99
30	290404 ± 0.28	292826 ± 0.58	30	290404 ± 0.28	292826 ± 0.58
40	377862 ± 0.10	381031 ± 0.65	40	377862 ± 0.10	381031 ± 0.65
50	487890 ± 0.33	494916 ± 1.38	50	487890 ± 0.33	494916 ± 1.38

Similarly the accuracy in determination of the assay of karanj oil sample was checked at five concentration levels i.e. 10, 20, 30, 40, 50µg/ml each in triplicate for 3 days and the percentage recoveries are recorded in table-2. % RSDs were calculated to measure the method reproducibility. Results indicated that the RSDs of all detected compounds were <1.5%, which indicated that the develop method had good reproducibility. The accuracy of the method was found to be good with the overall % RSD for recovery at known amount addition of 50µg, 100 µg and 150 µg levels were all within the limits. This indicates that the proposed method was found to be accurate.

Karanj oil Sample analysis

The assay of six different samples of *P. pinnata* and enriched karanj oil was subjected to methanolic extraction through ultrasonication method separately. Extract was filtered through 0.22µm PTFE syringe filter; thus the solution obtained was diluted to 10 times and used for analysis. Under the optimized chromatographic conditions the results obtained are represented in table-3. The corresponding overlay chromatogram of formulations with separation of peaks in seven batches as shown in figure-3. Results indicated that the UPLC

method had good precision, repeatability and recovery as shown above and that the developed assay was reliable and useful for assessing Karanj oil quality. The developed UPLC-PDA method was also reproducible, highly precise, and thus satisfactory for quantitative analysis.

Table 3. The content of karanjin and pongamol in karanj oil samples.

Sample code	Sample name	karanjin			pongamol		
		Retention time	Area	karanjin content found % w/w	Retention time	Area	pongamol content found % w/w
1 AKO	Market karanj oil	7.396	1092110	1.11	13.635	1289441	0.15
2 CPKO	Cold pressed karanj oil	7.377	565254	0.57	13.627	1002196	0.11
3 VKO	Vyas karanj oil	7.376	419464	0.43	13.63	511044	0.06
4 KNGS	Local seed samples	7.388	1072271	1.09	13.629	978765	0.11
5PH1	Pharmacy seeds sample 1	7.393	1921373	1.95	13.626	2390067	0.27
6 PH2	Pharmacy seeds sample 2	7.391	1561014	1.59	13.628	1360519	0.16
7 KEE	Karanj enriched oil	7.391	3409626	3.46	13.639	3964015	4.53

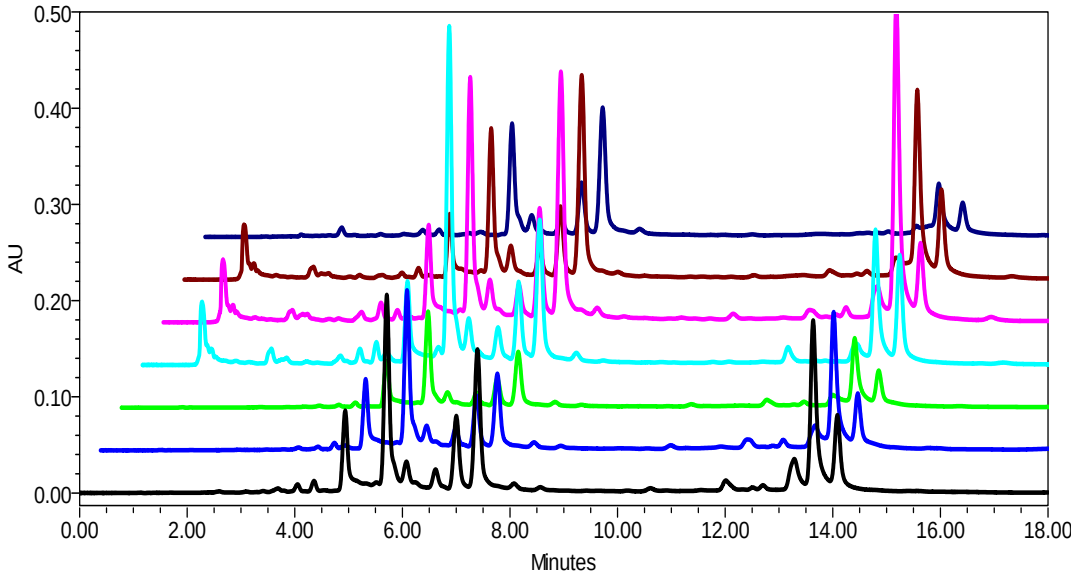

Fig. 3. Overlay chromatogram of (1-7) samples of karanj oil.

The amount of karanjin and pongamol in karanj oil were found in the range 0.43-1.95 %w/w and 0.06-0.27%w/w respectively (table-3). The karanjin and pongamol content in the enriched karanj oil was 3.46%w/w and 4.53 %w/w respectively (table-3, 7KEE).

CONCLUSION

Karanj oil possess antibacterial, antifungal properties for which it is used externally or topically on skin to treat or heal fungal skin diseases like itching and eczema etc. Karanj oil whether marketed or cold pressed and karanj seeds from pharmacy or from local region which were used in the study shows slight variation in their content of karanjin and pongamol assessed as shown above. Karanj oil sold commercially be used for medicinal purpose should contain 3-4 percent of karanjin and pongamol active principle for the efficacy. Thus suitable skin ointment formulations of karanj oil if made with enriched oil so as to have 3-4 % of karanjin and pongamol may have high potency. If pure karanjin is used in ointment that becomes an allopathic ointment which are not suitable to use topically and also to make unani or ayurvedic based herbal ointment. The study also describes a reversed-phase UPLC-PDA method was successfully developed and validated following ICH guidelines for the simultaneous detection of two active marker compounds the karanjin and pongamol in karanj oil. The study serves as a reference standard for the quality control check for karanj oil analysis.

ACKNOWLEDGMENT

The author thanks Director, CSIR-Indian Institute of Chemical Technology, Hyderabad for support and providing the necessary facilities and also thanks to Director General, CCRUM, New Delhi for giving constant encouragement and support. The author also thank Project Director, NIPER Hyderabad for providing the LCMS facility.

Conflict of interest: None

REFERENCES

1. The Unani Pharmacopoeia of India Part- I, Volume-I, Department of AYUSH, Ministry of Health and Family Welfare, Government of India, p. 48.
2. Tanaka T, Linuma M, Fuji Y, Yuki K, Mizuno M. Flavonoids in root and bark of *Pongamia pinnata*. Phytochemistry, 1992; 31: 993- 998.
3. Simin K, Ali Z, Khaliq-Uz-Zaman SM, Ahmad VU. Structure and biological activity of a new rotenoid from *Pongamia pinnata*, Natural Product Letters, 2002; 16(5): 351-357.

4. Pavanaram SK, Ramachandra Row L. New flavones from *Pongamia pinnata* (L.) Merr.: identification of compound D. Nature, 1955; 176: 1177.
5. Aneja R, Khanna RN, Seshadri TR. 6-Methoxyfuroflavone, a new component of the seeds of *Pongamia glabra*. Journal of the Chemical Society, 1963; p.163-168.
6. Warrier PK, Nambiar VPK, Ramakutty C. Indian Medicinal Plants. Vol. IV. Orient Longman Ltd., Madras, 1995; pp. 339.
7. Singh RK, Pandey BL. Anti-inflammatory activity of seed extracts of *Pongamia pinnata* in rats. Indian Journal of Physiology and Pharmacology, 1996; 40: 355-358.
8. Dahanukumar SA, Kulkarni RA, Rege NN. Pharmacology of medicinal plants and natural products. Indian Journal of pharmacology, 2000; 32: 81-118.
9. Prasad G, Reshmi MV. A manual of medicinal trees. Agrobios India. Propagation methods. Foundation for revitalization for local health tradition, India, 2003; pp.132.
10. Sridhar NB. Effect of dermovet ointment in treating dermatitis of domestic animals. Indian Journal of Animal Research, 2009; 43(3): 211-212.
11. Shejawal N, Menon S, Shailajan S. Bioavailability of karanjin from *Pongamia pinnata* L. in sprague dawley rats using validated RP-HPLC method. Journal of Applied Pharmaceutical Science, 2014; 4(3): 010-014.
12. Dharmesh SM, Vismaya, Belagihally SM, Rajashekhar S, Jayaram VB, Kanya S, *et al*., Gastroprotective properties of karanjin from karanja (*Pongamia pinnata*) seeds; role as antioxidant and H+, K+-ATpase inhibitor. Evidence-Based Complementary and Alternative Medicine, 2011; 20: 1-10.
13. Vismaya, Eipeson WS, Manjunatha JR, Srinivas P, Kanyaa TCS. Extraction and recovery of karanjin: A value addition to karanja (*Pongamia pinnata*) seed oil. Industrial Crops and Products, 2010; 32: 118–122.
14. Yadav RD,. Jain SK, Alok S, Prajapati SK, Verma A. *Pongamia Pinnata*: An overview. International Journal of Pharmaceutical Sciences and Research, 2011; 2(3): 494-500.
15. Lanjhiyana S, Patra KC, Ahirwar D, Rana AC, Garabadu D, Lanjhiyana SK. Karanjin in *Pongamia pinnata*: A novel Indian medicinal plant. Der Pharmacia Sinica, 2012; 3(1): 144-147.
16. Tamrakar AK, Yadav PP, Tiwari P, Maurya R, Srivastava AK. Identification of pongamol and karanjin as lead compounds with antihyperglycemic activity from *Pongamia pinnata* fruits. Journal of Ethnopharmacology, 2008; 118(3): 435-9.

17. Patel PP, Trivedi ND. Simple, efficient and economic method for isolation and analysis of karanjin and pongamol from karanja seed oil and screening of antimicrobial potential. International Journal of Pharmacy and Pharmaceutical Sciences, 2015; 7(7): 248-252.
18. Parmar BS, Gulati KC. Synergists for pyrethrins (II)-karanjin. Indian Journal of Entomology, 1969; 31: 239–243.
19. Shailajan S, Menon S, Pednekar S, Singh A. Wound healing efficacy of *Jatyadi Taila*: in vivo evaluation in rat using excision wound model. Journal of Ethnopharmacology, 2011; 138(1): 99-104.
20. Chopade VV, Tankar AN, Pande VV, Tekade AR, Gowekar NM, Bhandari SR, Khandake SN. International Journal of Green Pharmacy, 2010; 10: 72-75.
21. Gore VK, Satyamoorthy P. Determination of pongamol and karanjin in karanja oil by reverse phase HPLC. Analytical Letters, 2000; 33(2): 337-346.
22. Guideline on validation of analytical procedure-methodology. International Conference on Harmonization, Geneva, Switzerland, 1996.